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PRINCIPAL INVESTIGATOR: Susan E. Conrad, Ph.D.

CONTRACTING ORGANIZATION: Michigan State University
                           East Lansing, Michigan 48824

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Susan B. Conrad, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Michigan State University
East Lansing, Michigan 48824

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13. ABSTRACT *(Maximum 200 words)*

The goal of this work was to study the regulation of the thymidine kinase (TK) gene in non-tumorigenic and tumorigenic human breast epithelial cells. Previous studies in our laboratory had shown that this gene is regulated during the cell cycle by both transcriptional and post-transcriptional mechanisms. The approach used in the work reported here was to examine the expression of 2 hybrid genes. TK-Luc, which contains the human TK promoter linked to a luciferase reporter gene, was used to study transcriptional regulation. CMV-TK*, which contains an epitope-tagged human TK cDNA expressed from a constitutive CMV promoter, was used to study post-transcriptional regulation. These constructs were stably transfected into non-tumorigenic (184B5) and tumorigenic (MCF-7) human breast epithelial cells. No transfectants were obtained in the 184B5 cells, despite using several transfection protocols. The TK-Luc construct was expressed at low, constitutive levels in MCF-7 cells, indicating that the TK promoter fragment used was not sufficient to confer high, regulated levels of expression to linked genes in these cells. The CMV-TK* construct was regulated by serum, but not by estrogen/antiestrogen treatment, indicating that post-transcriptional regulation of this gene occurs in response to serum growth factors, and not as a function of cell cycle position.

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FOREWORD

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Susan E. Cons _2/1/98_
PI - Signature   Date

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INTRODUCTION

Background: The abnormal control of cell proliferation is a hallmark of all types of cancer, including breast cancer. The regulation of proliferation occurs largely during the G1 phase of the cell cycle. One important control point that occurs late in G1 is the restriction point, or "R" (1). Once past "R", cells are committed to proceed to S phase and complete the remainder of the cell cycle. Several molecules seem to be involved in the regulation of progression through "R". These include the products of tumor suppressor genes such as the retinoblastoma protein (pRB), transcription factors such as E2F, protein kinases such as the cdk kinases, and regulatory molecules known as cyclins (2-9). The activities of these molecules are critical in maintaining normal cell growth controls, since mutations in a number of them, including pRB and cyclin D1, are associated with oncogenic transformation (10,11). While a role for molecules such as pRB, cyclins and cdk kinases in controlling progression from G1 to S is certain, relatively little is known about their targets of action. One attractive model is that they regulate genes that are induced at G1-S. Studying the regulation of such genes therefore provides an attractive approach to studying the activities of cell cycle regulators. For a number of years, my laboratory has studied the expression of one such gene, the human thymidine kinase (TK) gene. TK enzyme levels are tightly regulated by both the growth state and cell cycle position of the cell; rapidly dividing cells (including tumor cells) contain high levels of activity while resting cells contain low or undetectable levels (12). During the cell cycle, TK activity is low in G1, increases sharply as cells enter S phase, and remains elevated throughout S and G2 (13,14). The increase in TK activity at G1-S is regulated by many of the same events that regulate entry into S phase and DNA synthesis (15), and has therefore been used as a model to study the events that regulate passage through "R".

Experiments in my laboratory and others have shown that the increase in TK enzyme activity at G1-S is the result of both transcriptional and post-transcriptional controls. In terms of transcriptional control, we demonstrated that TK mRNA levels parallel enzyme activity during mitogenic stimulation of quiescent cells; that is, they are low in G0 and G1 and highly induced as cells enter S phase (16,17). The fact that this increase in TK mRNA is due (at least in part) to activation of the TK promoter was shown in several ways. First, we demonstrated a 5-7 fold increase in the rate of transcription of the gene at G1-S in serum stimulated cells by nuclear run-on transcription assays (17). These results were later confirmed by other labs in additional cell lines (18). Second, we and others demonstrated that the human TK promoter confers G1-S regulation to linked heterologous genes (19-21). The sequences responsible for G1-S activation of the TK promoter have been mapped to a small region that contains binding sites for the cellular transcription factor E2F (19,22,23). E2F interacts with a number of cell cycle regulatory molecules including pRB, p107, cyclins A and E, and p33[cdk2] (7-9), and complexes containing several of these molecules have been reported to form with the TK promoter (24-26). Thus, TK transcription appears to be a direct target of cellular molecules that regulate entry into S phase, confirming our hypothesis that studying TK will give new insights into the mechanisms of action of these cell cycle regulators.

The 5-10 fold activation of the TK promoter at G1-S does not fully account for the much greater (up to 50-80 fold) increase in TK enzyme activity seen during the same interval, indicating that the gene is also regulated at a post-transcriptional level. In agreement with this, several years ago we and others demonstrated that enzyme levels are regulated independently of mRNA levels when the TK cDNA is expressed from a heterologous promoter (21,27). In our experiments, in

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which the human TK cDNA was expressed from the SV40 early promoter in transfected Rat3 (TK⁻) cells, mRNA levels were relatively constant during G1 and S phase, but TK enzyme levels were highly induced at G1-S, suggesting that the gene was regulated either translationally or post-translationally. In further experiments, we demonstrated that the increase of TK protein at G1-S in serum stimulated Rat3 cells is due to a 10 fold change in the stability of TK protein between G1 and S phase, and not to a change in the rate of translation of TK mRNA (28). Translational regulation of TK has been reported in several other cases, however, including at the G1-S transition in cycling HeLa cells (14) and during serum starvation and differentiation of murine cell lines (29-31). In addition, TK protein stability has been shown to be regulated at mitosis in cycling cells (14,32). Thus, the specific mechanisms that regulate expression of the TK gene may vary according to the specific cell type and/or growth conditions being studied.

Purpose of Work: The overall hypothesis underlying this work was that molecules such as pRB, cyclins and cdk kinases exert their effects on the cell cycle (at least in part) by altering the expression of specific genes at G1-S. We planned to test this hypothesis by examining the role of cell cycle regulators in controlling the expression of TK, a model G1-S activated gene. The objectives of the research were threefold: 1) To characterize the mechanisms (transcriptional, translational and post-translational) that regulate TK expression in non-transformed human breast epithelial cells, 2) To identify the cell cycle regulatory molecules involved in control of the TK gene in these cells and 3) To determine if regulation of TK (and therefore the function of cell cycle regulatory molecules) is perturbed in transformed cells compared to normal cells. By comparing TK regulation in non-transformed and transformed cells, we hoped to identify important changes in cell cycle regulation associated with oncogenic transformation.

Methods of Approach: The approach taken to address the objectives described above was to study the expression of hybrid genes in stably transfected human breast cell lines. To examine transcriptional and post-transcriptional regulation independently, we utilized hybrid genes containing either (A) the human TK promoter linked to a heterologous (luciferase) reporter gene or (B) an epitope-tagged human TK cDNA construct expressed from an unregulated (CMV) promoter. These constructs were transfected into non-transformed and transformed human breast epithelial cells, and their regulation during the cell cycle was examined.

BODY

Our original Statement of Work is given below. In the sections that follow, I will report our progress on each of these tasks.

Task 1: Determine mechanisms that regulate TK expression in non-transformed and transformed human breast epithelial cells.

> A) Construct hybrid genes to assay for transcriptional and post-transcriptional regulation.

> B) Establish stably transfected cell lines expressing hybrid genes.

> C) Study hybrid gene expression in stably transfected cell lines during mitogenic stimulation and during the cell cycle.

Task 2: Identify cell cycle regulatory molecules involved in TK gene expression.

> A) Prepare nuclear extracts and perform gel-shift assays with TK promoter fragments to determine proteins bound to the TK promoter control region.

> B) Perform co-immunoprecipitation experiments to assay for an association between TK protein and cell cycle regulatory molecules.

> C) Examine pattern of TK phosphorylation as a function of cell cycle position.

Task 1: Determine mechanisms that regulate TK expression in non-transformed and transformed human breast epithelial cells.

Task 1A: Construction of Hybrid Genes

The following hybrid genes were constructed for these studies.

1) TK-Luc: This plasmid contains a human TK promoter fragment (from -444 to +30 relative to the TK mRNA cap site) linked to the firefly luciferase (luc) coding sequences. Previous experiments in our laboratory and others have demonstrated that this TK promoter fragment confers G1-S regulation to heterologous genes in transfected rodent cells. This gene was used to study transcriptional regulation.

2) CMV-TK*: This plasmid contains an epitope tagged TK cDNA under the control of the constitutive CMV promoter. To construct this plasmid, two primers were designed and used to amplify the human TK coding sequences as shown in Figure 1. The 5' primer (a 62 mer) contained a Bam H1 site followed by an AUG initiation codon, the coding sequences for the HA1 epitope, and 20 bases (in frame) from the TK cDNA. The 3' primer (a 30 mer) contained an Eco R1 site linked to TK cDNA sequences 102 bp downstream of the TK stop codon. These primers were used to amplify TK cDNA sequences from p5'TK cDNA, a functional TK cDNA clone that we prepared and

have used previously (27). The PCR product was digested with Bam H1 and Eco R1, and subcloned into the vector pcDNA3 (In Vitrogen) which contains a CMV promoter for expression in mammalian cells. A clone containing the appropriate sized insert was isolated, and the entire insert was sequenced to insure that no mutations were introduced during the PCR amplification.

Task 1B: Establishment of Stably Transfected Cell Lines Expressing Hybrid Genes

Transfections were carried out with two human breast epithelial cell lines; 184B5, a non-tumorigenic cell line, and MCF-7, an estrogen dependent breast cancer cell line. We were unable to obtain stable transfectants in 184B5 cells, despite trying several different transfection reagents (lipofectin and superfect) and selectable markers (G418 [r] and hygromycin [r]). We did successfully isolate stable transfectants in MCF-7 cells, and report on our studies with these cell lines below.

i) Transfections using TK-Luc Reporter Gene: MCF-7 cells were co-transfected with TK-Luc DNA (5-10 µg/plate) and pSV2Neo (2 µg/plate) using lipofectin (Gibco/BRL). pSV2Neo contains a selectable G418[r] gene under the control of the SV40 early promoter. Stable transfectants were selected by growth in G418, and propagated as clonal cell lines. These cell lines were then assayed for expression of the luciferase reporter gene. Luciferase expression was low or undetectable in all of the cell lines obtained in this experiment. Furthermore, in cells expressing luciferase, it was not reproducibly regulated by serum or estrogen treatment (see below).

ii) Transfections using CMV-TK* Reporter Gene: MCF-7 cells were transfected with either the pCMV-TK* plasmid, or with the pcDNA3 vector, using lipofectin reagent. G418[r] colonies were selected, and propagated as clonal cell lines. Several of these cell lines were then assayed for expression of the transfected gene. The results of one such experiment are shown in Figure 2. Cytoplasmic extracts were prepared from two clonal cell lines (1C and 2G) containing pCMV-TK*, and one cell line (pcDNA3) containing the pcDNA3 vector. Twenty-five micrograms of protein from each extract were electrophoresed (in duplicate) on an SDS-polyacrylamide gel, blotted, and probed with antibodies to human TK (panel A) or to the influenza HA1 epitope (panel B). As shown in panel A, the pcDNA3 control cell line contains moderate levels of TK protein (which is expressed from the endogenous TK gene in MCF-7 cells). Both the 1C and 2G cell lines contain much higher levels of TK than the control, presumably because of the strength of the CMV promoter. As expected, the TK protein in cell lines 1C and 2G reacts with the anti-HA1 antibody (mAB 12CA5), while the endogenous TK protein in the control cell line does not. Thus, we succeeded in expressing an epitope tagged TK protein in these cells. This protein is enzymatically active, as indicated by the fact that both 1C and 2G cell lines contain approximately 10 fold higher TK activity than does the control cell line (data not shown).

Task 1C: Studies of Hybrid Gene Regulation during the Cell Cycle in Stably Transfected Cells.

We have carried out a series of experiments to study the effects of growth state and cell cycle position on the expression of the hybrid genes described above in MCF-7 cells. Two different experimental conditions were utilized; serum starvation/stimulation and estrogen depletion/addition.

i: Effects of serum and estrogen treatment on MCF-7 cell proliferation. We first attempted to establish conditions to synchronize MCF-7 cells by serum and estrogen depletion /addition.

MCF-7 cells require estrogen to proliferate, and in the absence of hormone they arrest in G1. To synchonize cells by estrogen depletion, they were plated in medium containing 5% FBS. On the following day, they were transferred to phenol-red-free medium containing 5% charcoal stripped serum (CSS) for 48-72 hrs. To assay for cell cycle position, nuclei were prepared, stained with propidium iodide, and analyzed for DNA content by flow cytometry. As shown in Table 1 below, approximately 92% of the cells were arrested in G1 after 2 days of estrogen depletion. In order to stimulate cells to re-enter the cell cycle and progress to S phase, fresh medium containing 10^{-9} M 17β-estradiol was added. As shown in the table, cells entered S phase beginning approximately 12 hrs after estradiol addition.

Table 1: Cell Cycle Analysis of MCF-7 Cells in the Presence and Absence of Estrogen

Sample	% G0/G1	%S	%G2/M
+ E	61.40	25.56	13.04
- E	92.76	4.74	2.50
2 hr, +E	93.55	5.40	1.05
6 hr, +E	92.53	4.53	2.94
12 hr, +E	89.76	7.18	3.05
18 hr, +E	49.56	46.99	3.45
24 hr, +E	32.84	49.58	17.57

Similar experiments were carried out to determine the effects of serum starvation and stimulation on the cell cycle of MCF-7 cells. Cells were plated in medium containing 5% FBS. The following day this medium was removed and replace with medium containing 0.2% serum for 2-3 days. At the end of the starvation, fresh medium containing 5% FBS was added, and cells were harvested at various time points and analyzed by flow cytometry as described above. No change in cell cycle distribution was seen at the various time points (data not shown), indicating that proliferation of these MCF-7 cells is not highly dependent upon serum growth factors.

ii: Effects of serum and estrogen on hybrid gene expression. We next carried out experiments to determine if the hybrid genes described above were regulated by either estrogen or serum in MCF-7 cells. The TK-Luc construct was not reproducibly regulated by either treatment, although we have previously shown that this construct is regulated by serum in normal rat fibroblasts (33). We do not understand the reason for this difference, especially because it has been shown that the endogenous TK promoter in MCF-7 cells is regulated by estrogen treatment (34). It may be that although the -444 bp/+30 bp TK promoter fragment used in these studies is sufficient to confer high levels of expression and serum regulation in normal rodent fibroblasts, additional sequences are required to obtain high and regulated expression in human breast cancer cells. In any case, due to our inability to demonstrate regulation in these stably transfected cell lines, this series of experiments were not pursued further.

In a second set of experiments, we examined serum and estrogen regulation of the pCMV-TK* reporter gene in the 1C and 2G cell lines desribed above. Since these are both subclones of MCF-7 cells, they were first assayed by flow cytometry to insure that their proliferation was still dependent on estrogen (data not shown). Once the estrogen dependence of these cells was confirmed, regulation of the transfected pCMV-TK* gene was examined in both serum stimulated and estrogen treated cells by Western blotting. Either MCF-7 cells or cells containing the pcDNA3 vector were also examined as a control for the regulation of the endogenous TK gene. Results of serum starvation/stimulation experiments are shown in Figure 3. The pCMV-TK* gene was regulated by serum in MCF-7 cells, with levels being low in serum starved cells and increasing within 4-6 hrs after serum stimulation. Northern blotting experiments showed that the mRNA from this gene was equivalent at all time points (data not shown), indicating that TK is regulated post-transcriptionally in human breast epithelial cells, either at the level of translation or protein stability. Somewhat surprisingly, this regulation was independent of cell cycle position since, as mentioned above, flow cytometric analysis showed no change in cell cycle position during the serum starvation/stimulation protocol. Thus, TK protein levels appear to be post-transcriptionally regulated in response to growth factor treatment, but not as a specific result of cell cycle position.

We have also examined post-transcriptional regulation of the pCMV-TK* gene in response to estrogen depletion and addition. The transfected cell lines were arrested by incubation for 2-3 days in either the absence of estrogen or presence of the antiestrogen ICI 182,780. The estrogen depleted cultures were then stimulated by the addition of B-estradiol and harvested at various time points. Representative results are shown in Figure 4. Surprisingly, TK* protein levels were not highly regulated by estrogen or antiestrogen (Figure 4A), although the endogenous TK gene in MCF-7 cells was highly regulated (Figure 4B). Although transcription of the endogenous TK gene in MCF-7 cells is regulated by estrogen, the epitope-tagged gene used in our studies is expressed from a constitutive (CMV) promoter, and we would only detect post-transcriptional regulation in this experiment. We therefore conclude that TK is not regulated on a post-transcriptional level in response to estrogen or antiestrogen treatment. Taken together with the results of the serum starvation/stimulation experiments described above, this suggests a two-stage model for TK regulation in MCF-7 cells. Transcription of the gene is regulated in response to cell cycle position, and is decreased under conditions of estrogen depletion or antiestrogen treatment, which arrest cells in G1. It is not directly regulated by the presence of serum growth factors, at least at the level of serum (0.2%) used in our experiments. Conversely, post-transcriptional regulation of the gene responds directly to serum growth factors. It does not occur specifically at the G1/S transition, and can occur in the absence of detectable effects on cell proliferation.

Task 2: Identify cell cycle regulatory molecules involved in TK gene expression.

Because of both time constraints (Task 1 taking longer than anticipated) and the results obtained in the experiments described in above, we did not pursue the experiments described in Task 2. Brief explanations are given below.

Task 2A: Preparation of Nuclear Extracts and Performing Gel-Shift Assays with TK Promoter.
Since we were unable to demonstrate regulation of a reporter gene in MCF-7 cells using the

-444/+30 bp TK promoter fragment linked to the Luc reporter gene, we did not proceed to identify the human proteins that bind to this fragment. Because the endogenous gene is regulated by estrogen, it is possible that we would see regulation using a longer promoter fragment. However, time constraints did not allow us to construct and test additional reporter genes.

<u>Tasks 2B and 2C: Studying Association of TK with other Proteins and TK Phosphorylation as a Function of Cell Cycle Position.</u>

A major goal of our work was to study post-transcriptional regulation of TK expression at the G1/S interface, in the hopes that it would allow us to identify novel regulatory molecules acting at this important control point in the cell cycle. However, our experiments showed that post-transcriptional regulation does not occur at G1/S in these cells, but rather as a direct response to serum growth factors. In addition, it is not directly linked to cell proliferation, since it occurs under conditions (0.2% serum) where proliferation is unaffected. We therefore did not pursue the mechanisms of post-transcriptional regulation further.

<u>New Task 3: Studies on TK Localization During the Cell Cycle.</u> The major goal of our work has been to characterize the mechanisms that regulate TK expression and activity at the G1/S boundary. Several years ago it was reported (using cell fractionation experiments) that the subcellular localization of TK varies during the cell cycle, with the protein being predominantly cytoplasmic during G1, and nuclear during S phase (35). In contrast, when we carried out immunofluorescence experiments on the human TK protein expressed in rat cells, we found it was cytoplasmic at all stages of the cell cycle (28). Recently, we became aware of reports that the ability to detect nuclear antigens by immunofluorescence is dependent upon the specific fixation and staining procedures used. We therefore initiated experiments to determine the subcellular localization of TK at different stages of the cell cycle in human breast epithelial cells. In a preliminary experiment, we used conditions where a high percentage of cells would be in S phase, since TK expression is expected to be high at that time. MCF-7 cells were plated on glass slides in medium containing 5% FBS. They were arrested by estrogen depletion, and then stimulated to re-enter the cell cycle by addition of B-estradiol. Sixteen hours after estradiol addition, the cells were fixed with paraformaldehyde, solubilized with Triton X-100, and then stained with anti-TK antibody followed by a fluorescent secondary antibody. They were then visualized by both light and confocal microscopy using a confocal microscope. The results of this experiment, which are shown in Figure 5, demonstrate that even at this time point, only a percentage of the cells express TK. Those that do show a fairly uniform staining of both the cytoplasm and nucleus, indicating that the enzyme is present in both subcellular compartments. We are currently in the process of extending these studies to different stages of the cell cycle, and to other cell types such as the non-tumorigenic 184B5 cell line, in order to determine if TK localization is regulated during the cell cycle, and if such regulation is disrupted during the process of oncogenic transformation.

CONCLUSIONS:

In conclusion, we have constructed hybrid genes to study transcriptional and post-transcriptional regulation of TK expression in human breast epithelial cells. This includes one construct (TK-Luc) designed to study transcriptional regulation and a second (CMV-TK*) designed to study post-transcriptional regulation. We were unable to obtain stable transfectants in non-

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tumorigenic (184B5) cells, although we tried several different transfection conditions. We have obtained stable transfectants in the estrogen dependent MCF-7 breast cancer cell line. Transfectants containing the TK promoter - Luc constructs were not regulated in these cells, although they are regulated in non-transformed rat cells. We also isolated and characterized several cell lines containing an epitope-tagged human TK protein expressed from a constitutive CMV promoter in order to study post-transcriptional regulation of the gene. Our results demonstrate that the gene is post-transcriptionally regulated in response to serum starvation/stimulation, but not in response to estrogen or antiestrogen treatment, although hormone treatment dramatically affect s the proliferative state of the cell. Thus, post-transcriptional regulation occurs in response to the presence/absence of serum growth factors, rather than as a result of cell cycle position.

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APPENDIX: SUSAN E. CONRAD

FIGURES 1-5

Figure 1:

BamH1

5' Primer

HA

AGCTGCATTAACCTGCCCAC

5'UTR

TKcDNA

3'UTR

TCCTTCAGCCCTCCGCACCTGC

3' Primer

EcoR1

Note: not drawn to scale.

B

pcDNA3 1C 2G

HA-TK→

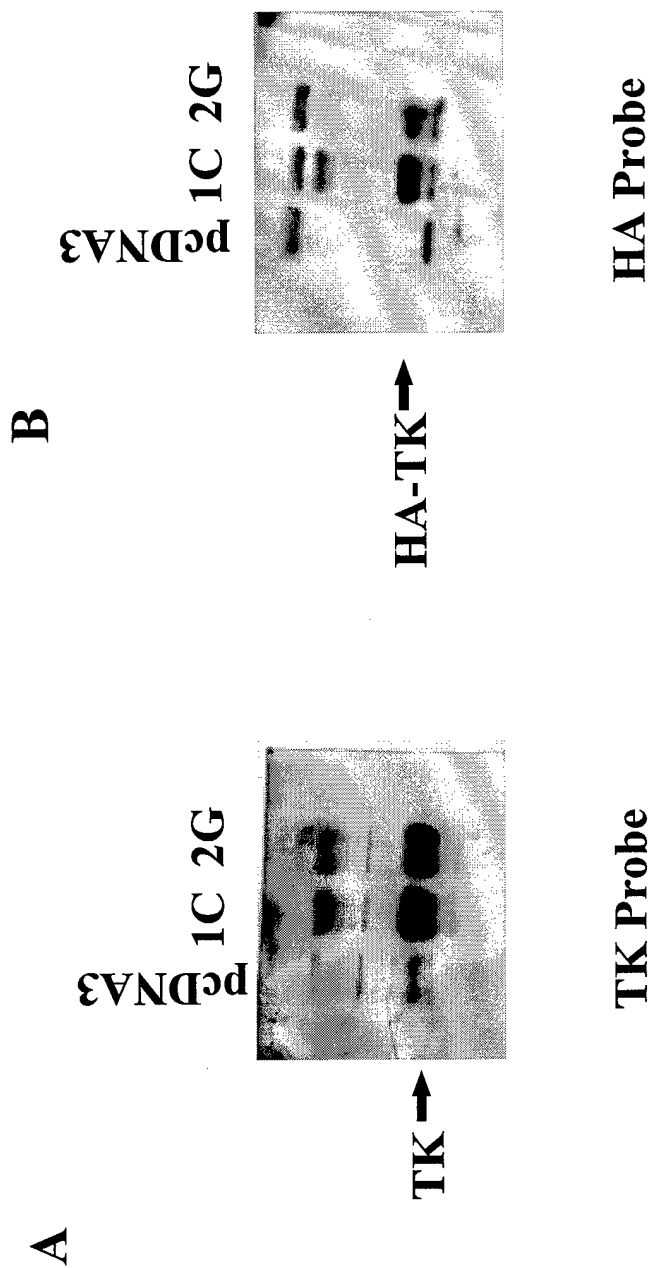HA Probe

A

pcDNA3 1C 2G

TK→

TK Probe

Figure 2: Western Blot Analysis of TK Expression in Transfected Cell Lines. Protein extracts were prepared from MCF-7 cell lines containing the pCMV-TK* gene (1C and 2G)or the pcDNA3 vector. They were then analyzed by Western blotting using either a TK (A) or HA (B) antibody probe.
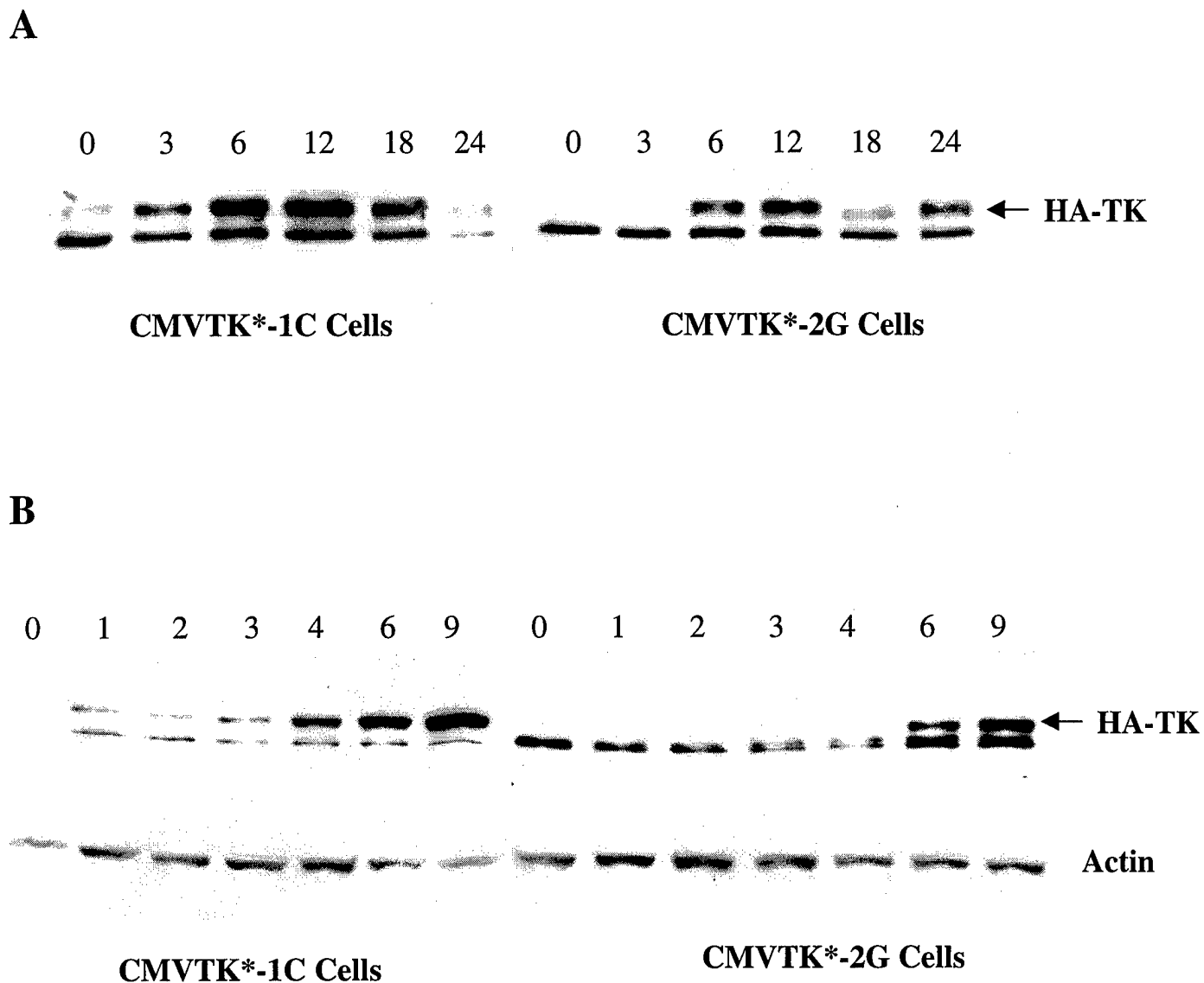
A

| 0 | 3 | 6 | 12 | 18 | 24 | 0 | 3 | 6 | 12 | 18 | 24 |

← HA-TK

CMVTK*-1C Cells   **CMVTK*-2G Cells**

B

| 0 | 1 | 2 | 3 | 4 | 6 | 9 | 0 | 1 | 2 | 3 | 4 | 6 | 9 |

← HA-TK

Actin

CMVTK*-1C Cells   **CMVTK*-2G Cells**

Figure 3: Regulation of Transfected TK Gene in Serum Stimulated MCF-7 Cells. Cells were serum starved and stimulated as described in the text. At the times indicated (hrs) after serum stimulation, cells were harvested, and extracts were prepared and analyzed for expression of the HA-tagged TK protein by Western blot analysis using an anti-HA antibody.

18

A

ICI 0 3 6 12 18 24

← HA-TK

CMV-TK* - 1C Cells

B

ICI 0 3 6 12 18 24

← Endogenous TK

← Actin
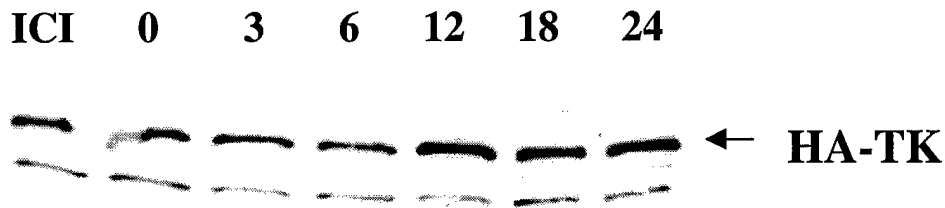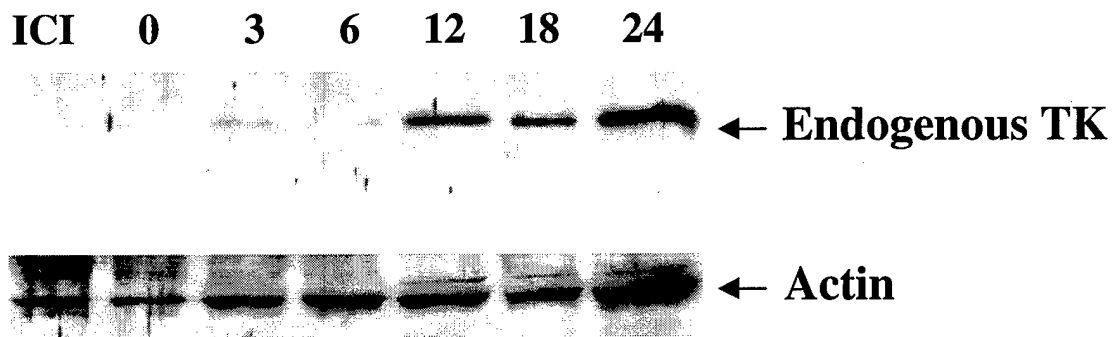
MCF-7 Cells

Figure 4: Regulation of Transfected and Endogenous TK genes during Estrogen and Antiestrogen Treatment of MCF-7 Cells. Cells were arrested by either estrogen depletion or ICI treatment for 2 days. At the end of this time, fresh medium containing estrogen was added to the estrogen depleted cultures. Cells were harvested at various times from 0 to 24 hr after estrogen addition, and extracts were prepared and analyzed for the levels of TK protein by Western blotting using anti-HA (A) or anti-TK (B) antibodies.
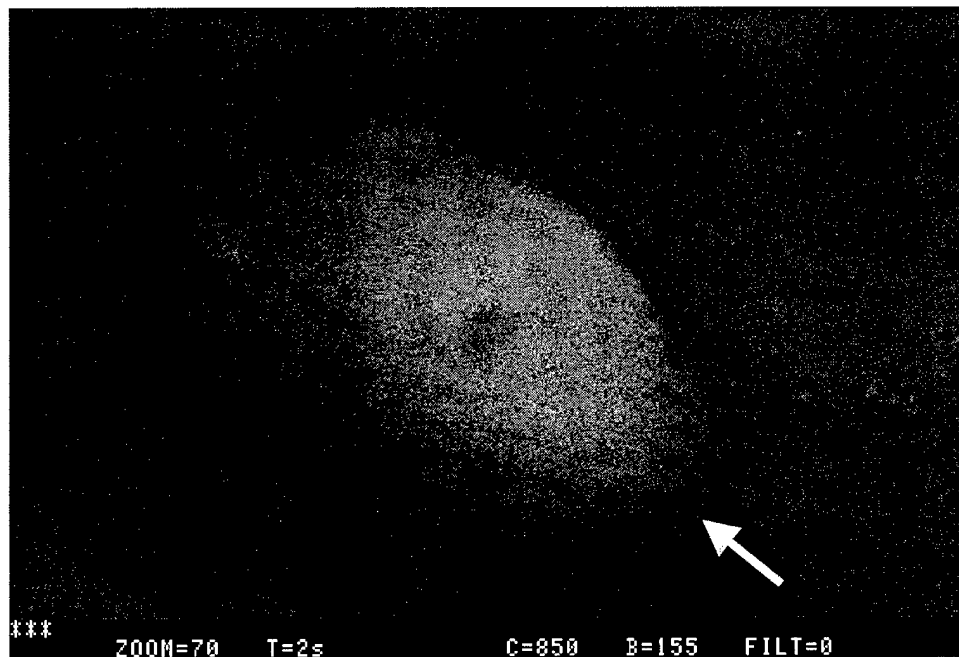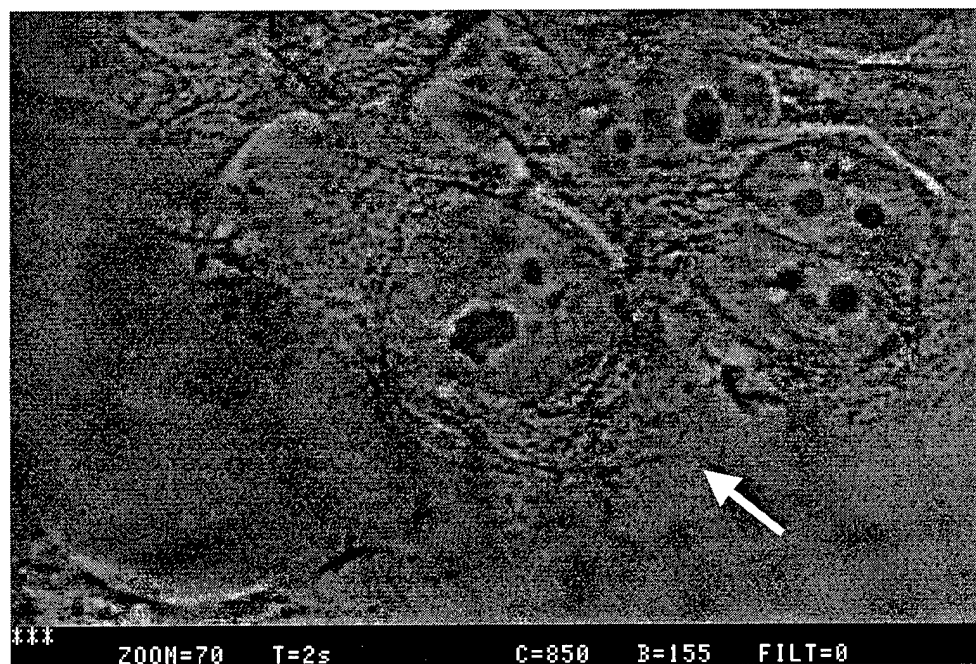
A


```
***   ZOOM=70   T=2s          C=850   B=155   FILT=0
```

B


```
***   ZOOM=70   T=2s          C=850   B=155   FILT=0
```

Figure 5: Immunofluorescence Staining of TK Protein in MCF-7 Cells. Cells were synchronized in G1 by estrogen depletion, and stimulated to re-enter the cell cycle by the addition of estrogen. Sixteen hours after estrogen addition, cells were fixed and treated with an anti-TK antibody followed by a fluorescent secondary antibody, and were visualized by confocal microscopy. A: Fluorescence microscopy. B: Light microscopy. The arrows point to a TK- expressing cell which demonstrates both nuclear and cytoplasmic fluorescence.

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Abstracts:

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Susan E. Conrad, Brandon Converse, Michael Deans, Joan Donnell, and Maria Pino.
The Department of Defense Breast Cancer Research Program Meeting
Era of Hope
Washington DC
October 31, 1997

Personnel Receiving Pay from this Effort:

Susan E. Conrad (PI)
Michelle Anderson (Graduate Assistant)
Brandon Converse (Research Assistant)
Michael Deans (Undergraduate Assistant)
Regina Laramee (Undergraduate Assistant)
Chia-Jen Liu (Graduate Assistant)
D. Nguyen (Undergraduate Assistant)
Maria Pino (Research Assistant)
Hemant Varma (Graduate Assistant)
Elizabeth Robinson (Undergraduate Assistant)